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Egg-albumen. We believe it is generally found that the preparation of crystalline egg-albumen by Hofmeister's method is a matter of some difficulty.

The process involves the following steps[1]:—

Fresh egg-white is thoroughly whipped into a froth and is then allowed to stand for 24 hours. It is then mixed with an equal bulk of saturated ammonium-sulphate solution and the mixture filtered from the precipitated globulins and mucoids. The filtrate is placed in shallow basins and is allowed to stand at the ordinary temperature. As evaporation proceeds the proteid separates in the form of globules of varying size. These are filtered off and re-dissolved, best in distilled water (Gabriel), and saturated ammonium sulphate solution is again added to the solution until a slight precipitate is formed. This is cleared up by the addition of a few drops of water and the solution is again allowed to stand in shallow dishes. The proteid again separates in the form of globules perhaps mixed with a few needles, but as a rule the process has to be repeated three or four times before any considerable proportion of needles is seen. Frequently it is found that the albumen remains obstinately in the form of globules and no true crystals can be obtained.

We have been able to make a modification in the process which, in itself of quite a minor character, has proved of the utmost importance in promoting the ease and rapidity of crystallization and in increasing the proportionate yield of the product. By introducing this slight modification we are able in fact to obtain large quantities of finely crystalline egg-proteid with great certainty, and in the course of twenty-four hours from the commencement of the operation.

[1] Hofmeister. *Zeitschr. f. physiol. Chem.* xiv. S. 165, 1892; Gabriel, *ibid.* xv. S. 459, 1891.

Upon adding to egg-white an equal bulk of saturated ammonium sulphate solution as directed by Hofmeister, the fixed alkali present in the proteid liberates a considerable quantity of ammonia, frequently in sufficient amount to produce a marked ammoniacal odour, and always enough to affect litmus-paper when this is held above the mixture.

In preparing products by Hofmeister's method we early came to the conclusion that this free alkali greatly interfered with the separation of crystals; and we found that after careful neutralization with acetic acid the mixture when exposed as usual in flat dishes yielded a crop of crystals much more rapidly and satisfactorily than when not so neutralised.

Further observations showed that, by working with a slight excess of acetic acid, the process becomes much easier still, and has the great advantage that absolutely no evaporation is required to produce crystals, while the yield is larger.

We have found that, working with fresh eggs, it is sufficient to add to the filtered mixture of egg-white and ammonium sulphate enough acetic acid to produce a slight permanent precipitate. It should be noticed that the very smallest excess begins to produce precipitation, and the acid should be diluted and added cautiously from a burette. It is well to wait between each addition so that a precipitate may be distinguished from the bubbles of gas which are evolved from carbonates present. When a faint permanent precipitate remains the mixture is left to stand in a closed vessel. In 24 hours or less a large proportion of the proteid will have separated in microscopic rosettes of acicular crystals quite unmixed with amorphous material, and this in the entire absence of evaporation, and therefore wholly without fear of contamination with ammonium sulphate crystals.

Even if sufficient acid be added to produce a fairly bulky precipitate, there is, as a matter of fact, no necessity to filter it off, for it is a remarkable fact that this precipitate, though amorphous at first, becomes rapidly crystalline on standing. As time elapses the amount greatly increases and finally the whole product is found to be uniformly crystalline[1]. The use of acetic acid in the manner we have described renders the preparation of crystalline egg-proteid quite the simplest of operations.

We shall best illustrate the exact details of the procedure which we

[1] Gabriel has shown that the globules obtained at the earlier stages of Hofmeister's process, on standing for some weeks in closed vessels with the mother-liquor may spontaneously change into needles. *loc. cit.*

have found so efficacious by describing the steps of a preparation as actually carried out in practice:

200 c.c. of egg-white obtained from new-laid fowls' eggs were mixed with an equal bulk of saturated ammonium sulphate solution, the latter being gradually added and the mixture stirred briskly with an egg-whisk between each addition. It was then allowed to stand over night. We find the preliminary whipping and standing of egg-white by itself recommended by Hofmeister, to be quite unnecessary, so that a day's delay may be avoided. But it is very essential that the membranes should be broken up at some stage in the process, and this is conveniently done by thorough churning during admixture with the salt. It is of advantage to allow the mixture to remain for a few hours before filtration. After standing the mixture was filtered, and, to the clear filtrate further saturated ammonium sulphate solution was added until a permanent precipitate was obtained, 16 c.c. being in this case necessary. Distilled water was then added from a wash bottle, a few drops at a time, until the solution was just again clear. It was then divided into two portions, each half being treated in a stoppered bottle with 10 % acetic acid, the acid being added drop by drop from a burette. To the first half sufficient acid was added to produce a slight but definite precipitate, 6·2 c.c. being required; to the second three or four drops were added in excess of this, being sufficient to produce a fairly bulky precipitate. The bottles were immediately closed, and 24 hours afterwards there was an abundance of precipitate in each. Examined microscopically under a $\frac{1}{6}$ objective both precipitates were found to be uniformly crystalline. In the case where least acid had been added the precipitate was in the form of beautifully regular rosettes of needles; in the other it consisted of the same mixed with sheaths and fan-shaped aggregates of needles, exactly like those figured by Halliburton in Schäfer's *Text-Book of Physiology*, I. p. 44. There was absolutely no trace of amorphous admixture in either case, and no globules. Such results as these we have never failed to obtain when fresh eggs have been employed, and others have successfully repeated the preparation from our description. The needles are always small but remarkably free from admixture.

It is generally considered necessary that perfectly fresh eggs should be used for the purpose of crystallization. We have found those ordinarily to be bought at the shops as new-laid give perfectly reliable results; and, although the degree of freshness undoubtedly affects the ease with which crystals are to be obtained, we have secured quite

satisfactory preparations from the cheapest eggs in the market, rejecting of course such as are obviously decomposed. Our observations seem to show that for some reason a somewhat greater excess of acid is desirable in the case of staler eggs, and it is better in all cases when the freshness of the egg-white is doubtful to add to the mixture with ammonium sulphate enough acetic acid to produce a fairly bulky precipitate and then to allow the whole without filtering to stand in a closed flask. If not fully crystalline in 24 hours it will certainly be so in 48. The following account of a preparation actually made will illustrate this:

200 c.c. of egg-white from cheap shop eggs were mixed under the conditions described above with an equal bulk of saturated ammonium sulphate solution, and, after 24 hours' standing, the mixture was filtered. The filtrate was strongly ammoniacal. Further ammonium sulphate was added until there was a definite precipitate, and this was re-dissolved by the cautious addition of water. It was then acidified by the addition of 10 % acetic acid drop by drop until a slight permanent precipitate remained (3·2 c.c. being required), and was put aside in a corked flask. After 24 hours the precipitate had scarcely increased (this is never the case with fresh eggs), so a further 0·25 c.c. of the dilute acid was added. This produced at once a considerable quantity of precipitate, which after another 24 hours had largely increased and was wholly crystalline.

Crystallization in closed or loosely corked flasks can always be obtained when acid is used, and is in every way preferable to evaporation in flat dishes. Other conditions being the same there is always a greater tendency during evaporation to the formation of globules instead of needles, and for the separation of a certain amount of amorphous precipitate. There is moreover always a liability for a scum of proteid to form on the surface of the fluid during evaporation which is often amorphous and difficult to separate from the crystalline precipitate. Without evaporation the crystals are always clean and free from amorphous admixture, while the yield is quite satisfactory and frequently very large. We have obtained as much as 40 % of the original proteid in crystalline form.

Recrystallization of the first product may be carried out with equal ease and with greater rapidity. If the first crop of needles be filtered off from the mother liquor and redissolved—it does not matter how little, nor, within reasonable limits, how much water is used to redissolve them—recrystallization can be obtained in a few hours. The

solution is made very faintly acid[1], with a few drops of the dilute acetic acid and some ammonium sulphate solution added until a faint turbidity is produced. On standing in a closed vessel rosettes of needles will deposit in course of the first 3 or 4 hours. In 24 hours a large proportion of the proteid will have recrystallized and will consist of pure needles. In the course of a week therefore the original products may be easily recrystallized five or six times.

All the indications go to show that the effect of acid in promoting crystallization is not due to any hydrolytic or other destructive effect upon the original proteid.

Whatever changes (if any) the contact with the strong saline fluid may produce in the original albumen before crystallization occurs[2], we believe that the acid adds nothing to these. The needles obtained, though in the absence of evaporation they are apt to separate in more regularly formed aggregates, are essentially the same as those obtained without the use of acid. It should be understood moreover that the results we have described are to be obtained with an extremely small excess of acid, so small that it is difficult to believe it capable of exerting any destructive influence.

The main influence of the acid is apparently in the direction of neutralizing free alkali, which is certainly inhibitory in its effects, and tends to retain the proteid precipitate in the globular form. It is possible that, on acidification, the proteid is liberated from some loose combination with the alkali; or it may be merely that the saline fluid becomes a better physical menstruum for the process of crystallization when it is neutral or acid than it is when alkaline.

But a secondary effect, requisite to explain the crystallization in closed vessels, is seen in the depression of solubility in the saline medium which the proteid undergoes when acid is in slight excess.

The mutual influence of ammonium sulphate and acetic acid in the precipitation of egg-albumen presents interesting quantitative aspects. As in the presence of a large proportion of the salt minute quantities of the acid may determine precipitation, so, in the presence of large

[1] If acid was used in the first stage there is no actual need to acidify in the second. Adding a very few drops of acid before the ammonium sulphate, however, markedly diminishes the amount of salt necessary to produce turbidity and induce crystallization (*vide infra*).

[2] We have ourselves obtained certain experimental results which suggest that Hofmeister's crystals consist of smaller molecular aggregates than the original amorphous proteid.

amounts of acid, very minute additions of the salt will throw the proteid out of solution.

It is of course well known that, in the presence of certain salts, acetic acid will precipitate albumens; but we believe it has not been observed in this connection how minute a quantity of ammonium sulphate will act as a precipitant of egg-albumen if acetic acid be first added in considerable quantity. Slight preliminary acidification of a solution will greatly reduce the quantity of salt necessary to precipitate it, and with increase of acid, within certain limits, the requisite amount of salt becomes less and less. The maximum influence of the acid is felt when about 30 % HA is present; after this proportion is exceeded, more salt becomes necessary for precipitation as the acidity increases, though the amount remains enormously less than in the absence of acid.

If to a one per cent. solution of egg-albumen sufficient glacial acetic acid be added for the solution to contain 30 % of the latter, subsequent admixture with at most 0·4 % of its bulk of saturated ammonium sulphate solution will produce visible precipitation, something like $\frac{1}{300}$th of the amount requisite when acid is absent. If it be mixed with 2 or 3 per cent. of its bulk of saturated ammonium sulphate solution precipitation in such an acid mixture becomes very nearly complete. These facts have an important bearing upon any theory of the influence of salts upon proteid precipitation. For instance, whatever the preliminary effect of the strong acid upon the albumen, the acid solution by itself of course remains perfectly clear, and it is very unlikely that the minute proportion of ammonium sulphate subsequently producing precipitation could involve any dehydrating effect.

For the purpose of this paper it is of chief interest to note that the precipitates produced by the small proportion of salt in the presence of a large amount of acid remain amorphous even on long standing; in marked contrast to those formed with a large proportion of salt and a very small proportion of acid, which, as we have shown, are obtained crystalline with extraordinary ease.

Serum Albumen. When horses' serum is mixed with saturated ammonium sulphate, as in Gürber's process for the preparation of crystals of serum albumen, there is, as in the case of egg-white, an abundant production of free ammonia; and, as with the ovalbumen, we have found that neutralization of the free alkali with acetic acid greatly assists crystallization. Acidification does not it is true lead to such remarkably rapid results as may be obtained with the egg-proteid;

but it greatly simplifies the process nevertheless, and enables crystals to be obtained from any specimen of horses' blood. Instead of using serum prepared from whipped and thoroughly centrifuged blood, which appears to be absolutely necessary when ammonium sulphate is employed alone, serum simply pipetted off after clotting may be employed, and excellent crystals may be obtained from oxalate plasma. With somewhat greater difficulty we have obtained fine crystals from dogs' serum.

The serum or plasma, freed from globulin by admixture with an equal bulk of saturated ammonium sulphate solution, and after filtering in the usual manner, is mixed with a further quantity of the salt solution until just turbid, the precipitate being redissolved by a minimal addition of water. Dilute acetic acid is then added very cautiously until a definite precipitate is again formed, and in this state the mixture is allowed to stand in closed flasks. The formation of crystals may not begin for some days, but within a week we have not failed to obtain a crop of needles, and the proportion of crystals to amorphous material continues to increase on standing. They are seen usually in preparations from horses' blood as aggregates shaped like palm leaves mixed with isolated needles of exceptionally large size.

On one occasion, working with ordinary serum pipetted from the clot, we evaporated in flat dishes after faintly acidifying. We then obtained crystals exactly agreeing in form with some kindly sent to us by Dr Sheridan Lea which he had himself prepared by Gürber's method. In our preparation the needles commenced to form within 24 hours, were abundant within 2 or 3 days, and from the first showed but slight admixture with amorphous material or globules; when once recrystallized they were quite pure.

We might add here that whenever evaporation is resorted to, so that some danger of confusing ammonium sulphate crystals with those of the proteid arises, it is useful to run a minute amount of such a stain as carbol-fuchsin or methylene blue under the cover-glass of a slide preparation. The proteid crystals take up the stain with great avidity and stand in marked contrast with the uncoloured ammonium sulphate crystals if such be present.